Potential Anti-Fibrotic Effect of Direct Acting Antiviral Drugs on CCl\textsubscript{4} Induced Hepatic Fibrosis in Rats

Alaa E. El-Sisi\textsuperscript{1} and Sherin Zakaria\textsuperscript{2}

\textsuperscript{1}Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tanta University, Tanta, Egypt
\textsuperscript{2}Department of Pharmacology and Toxicology, Faculty of Pharmacy, Kaferelsheikh University, Kaferelsheikh, Egypt

Corresponding Author: Sherin Zakaria; Sherin_zakaria@pharm.kfs.edu.eg

Dates: Received 7 January 2019; Accepted 18 March 2019

Editor: Helen Kwanashie

Copyright © 2019 Alaa E. El-Sisi and Sherin Zakaria. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract. Background: Hepatic fibrosis is a hall mark of chronic liver diseases such as chronic HCV. Direct acting antiviral (DAA) regimens such as Sofosbuvir (SOF) and daclatasvir (DAC) have been found to be associated with decreased fibrotic markers in HCV patients. It is not clear however the reported antifibrotic effect is antiviral dependent or not. Aim: This study investigated the effect of SOF and DAC in hepatic fibrosis induced by CCl\textsubscript{4} in rats. Method: Hepatic fibrosis was induced by (0.5 ml/kg) CCl\textsubscript{4} IP twice a week for six weeks. SOF (20 mg/kg/d) and DAC (30 mg/kg/d) were added in the last four weeks of treatments. Liver functions, fibrotic markers such as Hyaluronic acid and metalloproteinase-9 were detected using immunoassay. Liver tissues were examined by different stains. Results: SOF and DAC induced marked inhibitions in fibrotic markers expression significantly ($P \leq 0.001$). Moreover, the drugs protected liver tissues from progressed fibrosis. Conclusion: SOF/DAC antifibrotic effect is independent on its antiviral activity.

Keywords: sofosbuvir, daclatasvir, fibrosis, MMP-9, HA.

1. Introduction

Egypt is recognized as one of the hot spots that have high prevalence of hepatitis C virus (HCV) infection [1]. Chronic HCV infection is a major health problem. The disease progresses from fibrosis to cirrhosis within 20 years of infection [2]. Hepatic fibrosis is a wound healing response. Acute liver injury results in hepatocyte necrosis. The parenchymal cells then proliferate and replace the apoptotic hepatocytes [3]. This process of healing is associated with deposition of limited amount of collagen. During chronic injury this healing process is continued with much deposition of collagen and extracellular matrix [3]. The main cells producing collagen are hepatic stellate cells (HSCs) [4].

HCV infection promotes hepatic fibrosis either directly through its structural and non-structural proteins or indirectly through induction of immune response. HCV proteins target different molecular aspects that activate HSCs and trigger hepatic fibrogenesis [5, 6]. HCV proteins induce oxidative stress [7]. Reactive oxygen species induced by HCV are implicated in activation of profibrogenic cytokine such as transforming growth factor beta TGF-\(\beta\) as well as expression of extracellular matrix enzymes such as metalloproteinases [8].

The immune response against HCV infection plays an important role in activation of HSCs and its transition to myofibroblast through web of mediators secreted from infiltrated immune cells [9].
The approval of direct acting antiviral (DAA) is considered as a revolution in HCV eradication. Treating naive patients with ledipasvir/sofosbuvir (SOF) achieved sustained viral response (SVR) greater than 95% where daclatasvir (DAC) and SOF achieved SVR reaching up to (96% to 100%) [10, 11]. DAC and SOF combination therapy achieved SVR 84.54% in cirrhotic patients and 87.01% in treatment-experienced patients [12].

It has been reported that different DAA regimens are associated with decreased fibrotic markers. DAC and asunaprevir improved non-invasive fibrotic markers [13, 14]. Recent retrospective study involved HCV infected patients revealed that SOF/simeprevir, SOF/ribavirin, and SOF/DAC with or without ribavirin enhanced fibrosis scores and liver stiffness even in cirrhotic patients [15].

Although different studies [13–15] referred to the possible antifibrotic effect of DAA; it is not clear however the reported antifibrotic effect is antiviral dependent or independent. Moreover, the molecular targets for the proposed effect are not fully understood. We hypothesis that DAA in particular, SOF/DAC may retard hepatic fibrosis in a non-HCV fibrosis model. This study was designed to evaluate the possible antifibrotic activity of SOF/DAC in a non-HCV model.

2. Materials and Methods

2.1. Drugs and chemicals. Carbon tetrachloride CCl₄ was purchased from El Pharaoh (Cairo, Egypt). The CCl₄: corn oil solution [1:5, v/v] was prepared by adding 0.5 ml CCl₄ (neat) to 2 ml corn oil.

All other chemicals used herein were obtained from the El-Gomhouria company for trading chemicals and medical appliances (Cairo, Egypt) and were of the highest quality/analytical grade. SOF and DAC as powders were given as kind gifts from Marcyrl Company for pharmaceutical industries. For this study, DAC was dissolved in 0.9% normal saline solution while SOF was suspended in 0.2% methylcellulose.

2.2. Animals. Male Wister rats weighing 100-150 g were selected for this study. Rats were obtained from the National Research Center (Dokki, Giza, Egypt) and housed in a pathogen-free animals house in plastic cages with sawdust bedding. The facilities were maintained at 25 ±2°C, with ≈50% relative humidity, and 12-hr light: dark cycle. All rats had ad libitum access to standard rodent chow and filtered water. The Ethics Committee of Kaferelsheikh University approved study protocol involving animal use in this study. The experimental procedures were done in comply with the ARRIVE guidelines in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.3. Experimental protocol. A total of 65 rats were used in the study. All rats were injected by CCl₄ intraperitoneally (IP) (0.5 ml/kg) twice a week for six weeks [16]. At the 14th day of experiment 5 rats were killed by cervical dislocation for livers tissues collection. The rats were then allocated into four groups (10 rats each). The first group (CCl₄ group) was injected with CCl₄ as mentioned before without any protected agents for another four weeks. The second group of rats (CCl₄ + SOF group) was gavaged with SOF (20 mg/kg/d) [17] in addition to CCl₄ at the same starting dose. Rats in the third group (CCl₄ + DAC group) were given DAC (at 30 mg/kg/d) [18] by gavage and CCl₄ exactly as the same mentioned protocol. The fourth group (CCl₄ + SOF + DAC) of rats was received SOF (20 mg/kg/d), DAC (30 mg/kg/d) by gavage and CCl₄ as explained above. 10 rats were served as control ones (Control group). These rats were injected with corn oil (0.5 ml/kg) IP for two weeks then methylcellulose-saline were added to the corn oil for another 4 weeks. The last group of animals (SOF + DAC) were treated with same doses of SOF and DAC only for four weeks.

2.4. Preparation of blood sample and tissue homogenate. At 24 hr. after the final drug/vehicle gavage, all rats were anesthetized by diethylether then blood samples were immediately obtained via cardiac puncture and collected into uncoated tubes then allowed to be clotted at room temperature for 60 min. The samples were then centrifuged (3000gx, 10 min, 4°C). The resultant serum in each supernatant was recovered and stored at -20°C until analysis.

After the blood collection, the liver of each rat was carefully removed and weighed. Two 0.5-cm sections of the second largest lobe were fixed in 10% formol saline for histopathological examination. The remaining portions of each liver were divided into four parts weighed and individually stored at -80°C until analyzed. For those latter analyses, livers homogenates were prepared in 3 vol cold phosphate-buffered saline (PBS, pH 7.4). Each resulting mixture was centrifuged (6000gx, 20 min, 4°C) and aliquots of the derived supernatants were then used for biochemical analysis and measuring protein contents using standard kits (Wokea Medical Supplies, Changchun, China).

2.5. Indirect assay of liver functions. Serum alanine aminotransferase ALT and aspartate aminotransferase AST activity were each assayed using kits from Biodiagnostics (Giza, Egypt) that measure the amount of pyruvate and oxalo-acetate respectively, produced from 2,4-dinitrophenylhydrazine [19].

2.6. Liver fibrotic markers (hyaluronic acid HA and metalloproteinase-9 MMP-9). HA and MMP-9 levels in livers homogenates were determined using ELISA kits (Mybiosource, Southern California, USA). The detection levels for HA was 1.0 ng/mL and 0.1 ng/mL for MMP-9 assay.
2.7. Histopathological examination liver tissues. Autopsy samples were taken from liver of each rat in different groups and fixed in 10% formol saline for twenty-four hours. Washing was done in tap water then serial dilutions of alcohols (methyl, ethyl and absolute) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty-four hours. Paraffin bees wax tissues blocks were prepared for sectioning at 4μm thickness by microtome. The obtained tissues sections were collected on glass slides, deparaffinized then stained by hematoxylin & eosin stain for examination under light electric microscope [20].

2.7.1. Quantitative assessment of collagen accumulation in liver tissues. A second copy of slides were prepared from paraffin bees wax tissues blocks as mentioned above and stained with Masson's trichrome stain for detection of collagen deposition within tissues (colored as light blue). Images were quantitatively analyzed using image J-win 32. The spaces of collagen deposition areas were quantified in 3 films of each slide. Fibrosis areas were presented as mean of the three films.

2.7.2. Examination of HSCs activation. Alpha smooth muscle actin α-SMA was used as a marker of HSCs activation. Expression of α-SMA was evaluated using immunohistochemical techniques. Parallel sections of the liver tissues were stained using α-SMA antibodies (Sigma-Aldrich, St. Louis, MO, USA).

2.8. Grading of hepatic fibrosis. Fibrosis grading was done blindly at the end of treatment by two independent histopathologists according to Ishak classification of fibrosis and cirrhosis [21].

2.9. Statistics. All data are expressed as means ±SD. Statistical significance between outcomes from all groups was done using one-way analysis of variance (ANOVA) with Tukey’s test as a post-hoc test. A p value ≤0.05 was considered significant. Categorical variables were compared using Fisher’s exact test. Scoring data were compared using Mann-Whitney test. All analyses were performed using Prism software (v.6.0, Graphpad, San Diego, CA).

3. Results

3.1. Effect of SOF and DAC on liver function. CCl4 deteriorated liver functions significantly (P≤0.001) compared to control rats (ALT: 29.10 ± 4.73U/ml, AST: 34.18 ± 3.81U/ml). DAC partially inhibited the increased levels of ALT (134.3 ± 8.27) and AST (257.4 ± 14.38) levels compared to CCl4 treated rats (152.7 ± 17.58, 307.8 ± 27.7) respectively (P≤0.01). SOF showed nonsignificant changes in ALT as well as AST serum levels. Rats gavaged with both SOF and DAC showed significant changes in ALT and AST (141.3 ± 10.47, 271.5 ± 21.33) levels respectively compared to CCl4 treated rats while these changes were nonsignificant when compared to DAC group (Figure 1, I-A & I-B).

It to be noted that SOF/DAC combination in non CCl4 treated rats showed non-significant changes in liver functions (ALT: 31.3 ± 4.6, AST: 35.48 ± 2.98) compared to control rats (Figure 1, I-A & I-B).

3.2. SOF/DAC Retarded Fibrosis Progression

3.2.1. Effect of SOF/DAC on fibrotic markers. Hepatic fibrosis was assessed using HA as a marker of collagen accumulation in liver and MMP-9 as a marker of extracellular matrix dynamic change. Figure 1, II-B & II-C showed significant (P≤0.001) increases in HA (25.69 ± 2.66 ng/gm protein) and MMP-9 (18.20 ± 1.64 ng/gm protein) induced by CCl4 compared to control rats (2.51 ± 0.26, 2.31 ± 0.47).

Both SOF and DAC either used alone or in combination significantly (P≤0.001) dampened the levels of HA (19.20 ± 2.54, 6.04 ± 0.7, 7.28 ± 0.91) and MMP-9 (12.46 ± 1.27, 5.01 ± 0.68, 5.73 ± 0.94) respectively, compared to CCl4 rats. The antifibrotic activity of DAC was significantly (p≤0.001) more potent compared to SOF treated rats. It should be noted that combination therapy of the two drugs showed non-significant changes in the measured fibrotic markers when compared with DAC (Figure 1, II-B & II-C).

Rats treated with only SOF/DAC combination without CCl4 showed a non-significant changes in all measured fibrotic markers (HA 2.46 ± 0.36, MMP-9: 2.56 ± 0.3279) compared to control group. On the hand, results of this group were significantly lower (p≤0.001) compared to CCl4, SOF and even DAC concerning hyaluronic acid, MMP-9 and fibrotic area Figure 1 II-A, II-B & II-C.

3.2.2. Examination of liver tissues. Histopathological Examination of Rats’ Livers. Histopathological examination of liver tissues from control rats showed normal liver architecture of the hepatic parenchyma (Figure 2-A). On the other hand, liver tissues from rats injected with CCl4 for only 2 weeks showed focal hemorrhage in the parenchyma (Figure 2-B). The portal area showed periductal fibrosis surrounding the obliterated bile ducts (Figure 2-C). Upon examination of liver tissues from rats treated with CCl4 for six weeks, a highly thick and fibrotic Glisson’s capsule was clearly appeared (Figure 2-D). In addition, the portal area showed fibrosis and hyalinization with focal inflammatory cells infiltration and congestion in the portal vein (Figure 2-E).

SOF treated rats showed hyalinization of the collagen fibers and congestion in the portal vein (Figure 2-F) while liver section from DAC group showed fine fibrosis as well as dilatation in the portal vein (Figure 2-G). Similarly, rats treated with SOF and DAC showed only focal steatosis as well as fibrosis in the Glisson’s capsule (Figure 2-H). On the other hand, rats treated with only SOF/DAC...
Figure 1: Effect of SOF and DAC on liver enzymes and fibrotic markers: DAC (30 mg/kg/day orally) either alone or combined with SOF (20 mg/kg/day orally) showed significant inhibitions of ALT and AST induced by CCl₄ Figure 1-I-A&B. Figure 1-II-A illustrated the percent of fibrosis area calculated as collagen deposition spaces from three different films for each group of rats. Both drugs significantly inhibited expressions of HA (ng/gm protein) and MMP-9 Figure 1-II-B&C. Data are expressed as means ± SD. (n = 10). Value significantly different compared to 

\( ^a \) Control rats (p ≤ 0.05) \( ^{aaa}(p ≤ 0.001) \)

\( ^b \) CCl₄ treated rats (p ≤ 0.05) \( ^{bbb}(p ≤ 0.001) \)

\( ^c \) SOF treated rats (p ≤ 0.05) \( ^{ccc}(p ≤ 0.001) \)

\( ^d \) DAC treated rats (p ≤ 0.05) \( ^{ddd}(p ≤ 0.001) \).

Table 1: Effect of SOF and DAC on hepatic Fibrosis degrees in each group.

<table>
<thead>
<tr>
<th>Fibrosis grade</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCl₄</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCl₄+SOF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCl₄+DAC</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCl₄+SOF+DAC</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SOF+DAC</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

CCl₄ treated rates showed high grades of fibrosis. 90% of rats showed fibrosis at the 5th and 6th grade. SOF treated rats showed fibrosis ranged between the 3rd and 4th grade. DAC significantly prevent fibrosis progression. At least 80% of DAC treated rats expressed fibrosis at the 2nd grade. Data are expressed as numbers of rats in each fibrosis grade and its percentages. The significance was determined using the Mann–Whitney test. Data were significantly different compared to \(^a\) control rats, \(^b\) CCl₄ treated rats, \(^c\) SOF treated rats, \(n = 10\).

Collagen Precipitation within Liver Tissues and Fibrosis Grading. Liver sections from control rats as well as those treated with only SOF/DAC combination showed no fibrosis (Table 1) accordingly there wasn’t any collagen precipitated within the tissues (Figure 3-A & G). On the other hand, rats from CCl₄ groups showed sever fibrosis. Six rats showed cirrhosis and 3 rats showed grade five fibrosis (Table 1). The collagen deposition reached up to 59.2% ± 6.76 of film areas (Figure 3 II-A, Figure 3-C). SOF resulted in fibrosis grade ranged between 3rd and 4th grade (Table 1). The collagen fibers occupy about 21.59 ± 3.67% of the film areas (Figure 1 II-A, Figure 4-D). DAC significantly showed decrease in both fibrosis areas and fibrosis grading. Liver sections from those rats showed grade-2 fibrosis (Table 1). The collagen area appeared to be 6.89% ± 1.3 of total film area (Figure 1 II-A, Figure 4-E). Similarly, Liver sections from rats treated with SOF and DAC showed grade-2 fibrosis. Only one rat showed fibrosis of the 3rd degree (Table 1) and the fibrosis area was found to be only 9.2% ± 1.49 (Figure 1 II-A, Figure 4-F).
Figure 2: Gross pathology and histopathological examination of livers’ tissues by H&E stain: Liver extracted from control rats showed normal liver features such as reddish brown color, normal size, smooth surface and regular edges with smooth consistency (Figure 2-1). Gross examination of liver tissues from CCl₄ treated rats showed pale color and increased Glisson’s capsules thickness (red arrow) as well as hard consistency (Figure 2-2&3). Rats treated with SOF (20 mg/kg/day orally) showed only some fat dots (red arrow) and slightly enlargement in size with normal color and regular margins (Figure 2-4). Rats treated with either DAC (30 mg/kg/day orally) alone or in combination therapy showed normal shape and color with soft margins and smooth consistency (Figure 2-5&6). Figure 2-A demonstrates liver section from control rats showing normal architecture of the hepatic parenchyma. Liver sections from rats injected with CCl₄ for 2 weeks showing focal hemorrhage (black arrow) and periductal fibrosis (Figure 2-B&C). Examination of liver tissues from rats treated with CCl₄ for six weeks showed highly thick and fibrotic Glisson’s capsule (Figure 2-D) and fibrosis in portal area (black arrows) (Figure 2-E). Figure 2-F showed liver section from SOF demonstrating hyalinization of collagen fibers (black arrows) and congestion in the portal vein. Liver section of DAC group showed fine fibrosis (Figure 2-G). Similarly, SOF and DAC treated rats showed only focal steatosis as well as fibrosis in the Glisson’s capsule (black arrows) (Figure 2-H). Figure 2-I demonstrates liver section from SOF/DAC group showed normal liver architectures. The original microscopic magnification was 40×.

Activation of HSCs. Liver tissues were stained with α-SMA antibodies as a marker of HSCs activation. Liver sections from control group showed no reaction with α-SMA antibodies (Figure 4A) treating rats with CCl₄ for two weeks resulted in a mild reaction with α-SMA stain while six weeks of CCl₄ administration resulted in a strong positive reaction appeared in fibrous septa (Figure 4B&C). Liver section from SOF treating rats showed moderate reaction with the stain (Figure 4D). Liver section from rats treated with DAC whether alone or in combination with SOF showed only mild reaction with α-SMA antibodies (Figure 4E&F). Rats in SOF/DAC group showed nearly negative reaction with α-SMA stain.

4. Discussion

Hepatic fibrosis is a hall mark of chronic liver diseases [22]. One of the most important strategies to retard hepatic fibrosis progression is to eradicate causative agents of the chronic injury. According to this strategy, DAA regimens that inhibit HCV replication may have some beneficial effects on fibrosis regression. This proposal has been investigated in some clinical studies that were designed to screen the DAA antifibrotic effect. These studies revealed that different HCV treatment regimens such as SOF/simeprevir and SOF/DAC improved fibrosis scores measured by different non-invasive fibrotic markers [13–15].
The present study proposed that the reported antifibrotic effect may be independent of HCV eradication. It investigated the effect of SOF/DAC on CCl₄ induced hepatic fibrosis in rats. The used DAA inhibited CCl₄ induced HA and significantly dampened the precipitation of collagen fibers in liver tissues. The drugs decreased spaces of the fibrosis areas as well as fibrosis grades. The drugs also inhibited HSCs activation.

Both drugs retarded fibrosis progression however DAC was more potent compared to SOF. Moreover, addition of SOF to DAC resulted in a nonsignificant antifibrotic effect when compared to daclatasvir.

SOF and DAC doses were selecting in guidance with previous preclinical studies that reported the antiviral SOF dose in animal studies as a range between 20 and 80 mg/kg/day [16, 23] where DAC dose that have antiviral effect in animals was 30 mg/kg/day [17].

It is not clear whether increasing SOF dose may potentiate its effect or not. Studies at different doses levels including therapeutic and subtherapeutic doses of both drugs may be needed to optimize the antifibrotic potency of each drug alone or in combination.

In support of the present results, Mazzarelli and his colleagues [24] reported declines in fibrotic markers exactly HA, TGF-beta and interleukin-6 IL-6 in cirrhotic patients treated with ledipasvir and daclatasvir. They also pointed out that HA is highly sensitive to the change in fibrotic stages. Data that referred to antifibrotic effect of these drugs in non HCV patients are limited. Recently, it has been reported that human equivalent doses of SOF and DAC inhibited liver injury induced by thioacetamide [23].

The present study revealed that SOF and DAC whether used alone or in combination inhibited the release of MMP-9 induced by CCl₄. MMP-9 is one of the gelatinases that induced in all hepatic fibrosis models [25]. Its expression is associated with progressed inflammation. In addition, it is recognized as one of the profibrogenic cytokine TGF-β inducers [26, 27]. Moreover, MMP-9 Plays a pivotal role in HSCs activation and fibrogenesis in different fibrosis models.
Figure 4: Effects of SOF and DAC on CCl₄ induced HSCs activation detected by α-SMA. Liver section from control group showed no reaction with α-SMA antibodies (Figure 4A). Treating rats with CCl₄ for two weeks resulted in mild reaction with α-SMA stain while six weeks of CCl₄ administration resulted in strong positive reaction appeared as brown color in fibrous septa (Figure 4B&C). Figure 4D, E and F demonstrated liver sections from SOF, DAC and combination of both drugs respectively showing milder degree of stain interaction. Figure 4G showed liver section from rats in SOF/DAC group showed nearly negative reaction with α-SMA stain. Dark blue arrows showed the areas of positive reaction with α-SMA antibodies. The original microscopic magnification was 40×.

[28]. It has been reported that MMP-9 is detected in HSCs and activated by 3D type I collagen [29]. It is also reported that activated HSCs from human liver biopsies expressed MMP-9 [28].

These data supported that MMP-9 may be one of the proposed mechanisms implicated in the antifibrotic effect of SOF and DAC.

In conclusion, SOF and DAC antifibrotic may be antiviral independent. DAC was more potent than SOF. Eventually addition of SOF did not significantly improve DAC antifibrotic activity. At cellular level, these drugs inhibited HSCs activation and decreased collagen accumulation in liver tissues.

This study raised some questions that needs further investigations. For example, the optimum dose for antifibrotic activity of each drug and detailed molecular targets that may modulated by this combination.

Competing Interests

The authors declare no competing interests.

Acknowledgment

The authors are grateful to Prof. Adel Bakeer (Professor of Pathology, Cairo University) for the kind help in performing the histopathology, histochemical and immunohistochemical staining of liver sections as well as interpretation of those results.

References


